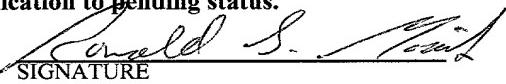


FORM PTO-1390 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE (MODIFIED)		ATTORNEY'S DOCKET NUMBER X-9872
<b>TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371</b>		U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5)
INTERNATIONAL APPLICATION NO. PCT/US97/01978	INTERNATIONAL FILING DATE 02/06/1997 (06.02.97)	PRIORITY DATE CLAIMED 02/06/1996 (06.02.96)
TITLE OF INVENTION <b>DIABETES THERAPY</b>		JUN 16 1998 SCAO [Circular stamp: PCT/PTO/US/TRADEMARKS]
APPLICANT(S) FOR DO/EO/US Borts, Tracy L., et al.		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.		
2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.		
3. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).		
4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.		
5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))		
a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).		
b. <input type="checkbox"/> has been transmitted by the International Bureau.		
c. <input checked="" type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).		
6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).		
7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))		
a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).		
b. <input type="checkbox"/> have been transmitted by the International Bureau.		
c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.		
d. <input checked="" type="checkbox"/> have not been made and will not be made.		
8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).		
9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).		
10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).		
<b>Items 11. to 16. below concern document(s) or information included:</b>		
11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.		
12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.		
13. <input type="checkbox"/> A FIRST preliminary amendment.		
14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.		
15. <input type="checkbox"/> A substitute specification.		
A change of power of attorney and/or address letter.		

16.	Other items or information:																		
17.	<input checked="" type="checkbox"/> The following fees are submitted:	<b>CALCULATIONS PTO USE ONLY</b>																	
<b>BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):</b> Search Report has been prepared by the EPO or JPO.....\$930.00  International preliminary examination fee paid to USPTO (37 CFR 1.482) .....\$720.00  No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))....\$790.00  Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....\$1070.00  International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....\$98.00																			
<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>		\$720.00																	
Surcharge of \$130.00 for furnishing the oath or declaration later than <u>20</u> <u>30</u> months from the earliest claimed priority date (37 CFR 1.492(e)).		\$0																	
<table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th>CLAIMS</th> <th>NUMBER FILED</th> <th>NUMBER EXTRA</th> <th>RATE</th> </tr> </thead> <tbody> <tr> <td>Total claims</td> <td>18-20=</td> <td>0</td> <td>X \$22.00 \$0</td> </tr> <tr> <td>Independent claims</td> <td>5-3=</td> <td>2</td> <td>X \$82.00 \$164.00</td> </tr> <tr> <td>MULTIPLE DEPENDENT CLAIM(S) (if applicable)</td> <td></td> <td></td> <td>+ \$270.00 \$0</td> </tr> </tbody> </table>		CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	Total claims	18-20=	0	X \$22.00 \$0	Independent claims	5-3=	2	X \$82.00 \$164.00	MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$270.00 \$0		
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Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).		\$0																	
<b>SUBTOTAL =</b>		\$884.00																	
Processing fee of \$130.00 for furnishing English translation later than <u>20</u> <u>30</u> months from the earliest claimed priority date (37 CFR 1.492(f)).		\$0																	
<b>TOTAL NATIONAL FEE =</b>		\$0																	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +		\$0																	
<b>TOTAL FEES ENCLOSED =</b>		\$884.00																	
		Amount to be refunded	\$																
		charged	\$																
a. <input type="checkbox"/>	A check in the amount of \$ _____ to cover the above fees is enclosed.																		
b. <input checked="" type="checkbox"/>	Please charge my Deposit Account No. 05-0840 in the amount of \$884. to cover the above fees. A duplicate copy of this sheet is enclosed.																		
c. <input checked="" type="checkbox"/>	The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 05-0840. A duplicate copy of this sheet is enclosed.																		
<b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b>																			
<b>SEND ALL CORRESPONDENCE TO:</b> <b>ELI LILLY AND COMPANY</b> <b>PATENT DIVISION/RSM</b> <b>LILLY CORPORATE CENTER</b> <b>INDIANAPOLIS, INDIANA 46285</b>  <u>12/June/98</u>		 <b>SIGNATURE</b> Ronald S. Maciak <b>NAME</b> 35,262 <b>REGISTRATION NUMBER</b> <b>TELEPHONE: (317) 276-1664</b>																	

PTO/PCT Rec'd 16 JUN 1998

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DIABETES THERAPY

The present invention relates to gene therapy and provides stable, transformed mammalian cell lines and vectors useful as vehicles for transferring functional DNA sequences 5 whose protein products are useful in the treatment of *diabetes mellitus*.

The human hormone glucagon is a 29-amino acid hormone produced in pancreatic A-cells. The hormone belongs to a multi-gene family of structurally related peptides that 10 include secretin, gastric inhibitory peptide, vasoactive intestinal peptide and glicentin. These peptides variously regulate carbohydrate metabolism, gastrointestinal mobility and secretory processing. However, the principal recognized actions of pancreatic glucagon are to promote hepatic 15 glycogenolysis and gluconeogenesis, resulting in an elevation of blood sugar levels. In this regard, the actions of glucagon are counter regulatory to those of insulin and may contribute to the hyperglycemia that accompanies *diabetes mellitus* (Lund, P.K., et al., *Proc. Natl. Acad. Sci. U.S.A.*, 20 79:345-349 (1982)).

When glucagon binds to its receptor on insulin producing cells, cAMP production increases which in turn stimulates insulin expression (Korman, L.Y., et al., *Diabetes*, 34:717-722 (1985)). Moreover, high levels of 25 insulin down-regulate glucagon synthesis by a feedback inhibition mechanism (Ganong, W.F., *Review of Medical Physiology*, Lange Publications, Los Altos, California, p. 273 (1979)). Thus, the expression of glucagon is carefully regulated by insulin, and ultimately by serum glucose levels.

Preproglucagon, the precursor form of glucagon, is 30 encoded by a 360 base pair gene and is processed to form proglucagon (Lund, et al., *Proc. Natl. Acad. Sci. U.S.A.* 79:345-349 (1982)). Patzelt, et al. (*Nature*, 282:260-266 (1979)) demonstrated that proglucagon is further processed 35 into glucagon and a second peptide. Later experiments demonstrated that proglucagon is cleaved carboxyl to Lys-Arg

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or Arg-Arg residues (Lund, P.K., et al., Lopez L.C., et al., *Proc. Natl. Acad. Sci. U.S.A.*, 80:5485-5489 (1983), and Bell, G.I., et al., *Nature* 302:716-718 (1983)). Bell, G.I., et al., also discovered that proglucagon contained three discrete and highly homologous peptide regions which were designated glucagon, glucagon-like peptide 1 (GLP-1), and glucagon-like peptide 2 (GLP-2). Lopez, et al., demonstrated that GLP-1 was a 37 amino acid peptide and that GLP-2 was a 34 amino acid peptide. Analogous studies on the structure of rat preproglucagon revealed a similar pattern of proteolytic cleavage at Lys-Arg or Arg-Arg residues, resulting in the formation of glucagon, GLP-1, and GLP-2 (Heinrich, G., et al., *Endocrinol.*, 115:2176-2181 (1984)). Finally, human, rat, bovine, and hamster sequences of GLP-1 have been found to be identical (Ghilione, M., et al., *Diabetologia*, 27:599-600 (1984)).

The conclusion reached by Lopez, et al., regarding the size of GLP-1 was confirmed by studying the molecular forms of GLP-1 found in the human pancreas (Uttenthal, L.O., et al. *J. Clin. Endocrinol. Metabol.*, 61:472-479 (1985)). Their research showed that GLP-1 and GLP-2 are present in the pancreas as 37 and 34 amino acid peptides respectively.

The similarity between GLP-1 and glucagon suggested to early investigators that GLP-1 might have biological activity. Although some investigators found that GLP-1 could induce rat brain cells to synthesize cAMP (Hoosein, N.M., et al., *Febs Lett.* 178:83-86 (1984)), other investigators failed to identify any physiological role for GLP-1 (Lopez, L.C., et al. *supra*). The failure to identify any physiological role for GLP-1 caused some investigators to question whether GLP-1 was in fact a hormone and whether the relatedness between glucagon and GLP-1 might be artifactual.

It has now been shown that biologically processed forms of GLP-1 have insulinotropic properties and delay gastric emptying. GLP-1(7-34) and GLP-1(7-35) are disclosed in U.S. Patent No: 5,118,666, herein incorporated by

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reference. GLP-1(7-37) is disclosed in U.S. Patent No: 5,120,712, herein incorporated by reference.

Variants and analogs of GLP-1 are known in the art. These variants and analogs include, for example, GLP-1(7-36), Gln<sup>9</sup>-GLP-1(7-37), Thr<sup>16</sup>-Lys<sup>18</sup>-GLP-1(7-37), and Lys<sup>18</sup>-GLP-1(7-37). Derivatives of GLP-1 include, for example, acid addition salts, carboxylate salts, lower alkyl esters, and amides (see, e.g., WO91/11457). Generally, the various disclosed forms of GLP-1 are known to stimulate insulin secretion (insulinotropic action) and cAMP formation (see, e.g., Mojsov, S., *Int. J. Peptide Protein Research*, 40:333-343 (1992)).

More importantly, numerous investigators have demonstrated a predictable relationship between various *in vitro* laboratory experiments and mammalian, especially human, insulinotropic responses to exogenous administration of GLP-1, GLP-1(7-36) amide, and GLP-1(7-37) acid (see, e.g., Nauck, M.A., et al., *Diabetologia*, 36:741-744 (1993); Gutniak, M., et al., *New England J. of Medicine*, 326(20):1316-1322 (1992); Nauck, M.A., et al., *J. Clin. Invest.*, 91:301-307 (1993); and Thorens, B., et al., *Diabetes*, 42:1219-1225 (1993)).

The fundamental defects responsible for causing hyperglycemia in mature onset diabetes include impaired secretion of endogenous insulin and resistance to the effects of insulin by muscle and liver tissue (Galloway, J.S., *Diabetes Care*, 13:1209-1239, (1990)). The latter defect results in excess glucose production in the liver. Thus, whereas a normal individual releases glucose at the rate of approximately 2 mg/kg/minute, a patient with mature onset diabetes releases glucose at a rate exceeding 2.5 mg/kg/minute, resulting in a net excess of at least 70 grams of glucose per 24 hours.

Because there exists exceedingly high correlation between hepatic glucose production, fasting blood glucose levels, and overall metabolic control as indicated by glycohemoglobin measurements (Galloway, J.A., *supra*; and Galloway, J.A., et al., *Clin. Therap.*, 12:460-472 (1990)), it

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is readily apparent that control of fasting blood glucose is essential for achieving overall normalization of metabolism sufficient to prevent hyperglycemic complications. Since existing insulin therapies rarely normalize hepatic glucose production without producing significant hyperinsulinemia and hypoglycemia (Galloway, J.A., and Galloway, J.A., et al., *supra*) alternative approaches for diabetic therapy are needed.

Therapy based on administration of longer acting GLP-1 analogs is one such approach. To date however, this approach has failed to deliver long term efficacious doses to individuals due in large part because the serum half-life of GLP-1(7-37) is quite short. Therefore, the quest for alternative approaches continues.

Gene therapy offers a new avenue for treating diseases rooted in hormone deficiencies because it operates as an *in vivo* protein production and delivery system. This is an especially attractive approach since gene therapy also offers the possibility of physiologically regulating the production and secretion of proteins in response to homeostatic mediators within the body.

Gene therapy can be effected in a number of ways. Retroviral-mediated gene transfer was suggested for treating human diseases involving malfunctioning bone marrow. Anderson *et al.*, *Science* 226: 401 (1984). In addition, PCT Publication Number WO93/09222 (May 13, 1993) and U.S. Patent Number 5,399,346 (March 21, 1995) disclose the genetic alteration of primary human cells that are cultured then reintroduced into the body for the treatment of a variety of diseases.

Many heritable diseases such as diabetes result from the absence of a functional gene necessary to provide the animal with an adequate supply of a vital protein. The goal of gene therapy is to deliver a nucleic acid sequence, present on an RNA or DNA vector, which is capable of encoding the desired, therapeutic protein. Although a number of methods exist to deliver the DNA or RNA vector containing the

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desired nucleic acid sequence into the target mammalian cells, two procedures termed *ex vivo* and *in vivo* are generally employed.

Ex vivo gene therapy consists of four primary  
5 steps: (1) Primary cells (target cells) are removed from the individual in need of therapy; (2) The gene therapy nucleotide sequence is incorporated into the target cell *in vitro*; (3) The transformed cells expressing the incorporated nucleotide sequence encoding the protein of interest are  
10 identified, isolated, and expanded; and (4) The transformed cells are reintroduced into the individual.

Ex vivo therapy generally results in the incorporation of the nucleotide sequence encoding the protein of interest into the chromosomal DNA of the target cell. The  
15 critical step of ex vivo gene therapy is the proper introduction of the nucleotide sequence encoding the desired protein into the target cell. This transfer of DNA can be accomplished by a number of well documented methods such as: calcium phosphate precipitation, electroporation, and  
20 adenoviral or retroviral vectors (Current Protocols in Molecular Biology, John Wiley and Sons, 1989; Methods of Cell Biology, 43: 161 - 189, 1994; Proc. Natl. Acad. Sci. USA 85: 6460 - 6464, 1985); although, other well known methods are also consistent with this invention.

In vivo gene therapy generally describes the transfer of a desired nucleic acid sequence, located on a transport vector, directly into an individual in need of therapy. This gene therapy approach does not require that the target cells be first removed and manipulated *in vitro*.  
25 Once the vector containing the desired nucleotide sequence is introduced into the individual, the vector moves into the nucleus of the target cell and the nucleotide sequence of interest integrates into the chromosomal DNA of the target cell with varying degrees of efficiency. A number of well documented methods exist for introducing nucleic acids into an individual requiring therapy such as direct injection of  
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DNA and the use of recombinant viral vectors (Gene Therapy, A Handbook for Physicians, Mary Ann Liebert, Inc., 1994).

The present invention provides a method of treating both Type I and Type II diabetics through a different gene therapy approach. A stable mammalian cell line is transformed by a nucleic acid vector such that it secretes a GLP-1(7-37)-based protein, as defined by SEQ ID NO 1, followed by implantation into an individual needing treatment. Once implanted, the GLP-1(7-37)-based protein, in conjunction with high serum glucose levels, causes pancreatic beta cells to produce insulin in non-insulin dependent *diabetes mellitus* (NIDDM) patients and delays gastric emptying in both NIDDM and insulin dependent *diabetes mellitus* IDDM patients.

Accordingly, one embodiment of this invention provides a method of treating Type I or Type II diabetes in a mammal in need thereof comprising implanting a cell line transformed with a vector comprising a promoter driving expression of a DNA sequence encoding a protein of SEQ ID NO 1

His-Xaa<sup>1</sup>-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-  
Leu-Xaa<sup>2</sup>-Gly-Gln-Ala-Ala-Xaa<sup>3</sup>-Xaa<sup>4</sup>-Phe-Ile-Ala-Trp-Leu-  
Val-Lys-Gly-Arg-Xaa<sup>5</sup> (SEQ ID NO 1)

wherein

Xaa<sup>1</sup> is Ala, Gly, Val, Thr, and Ile;  
 Xaa<sup>2</sup> is Glu, Gln, Ala, Thr, Ser, and Gly;  
 Xaa<sup>3</sup> is Lys, and Arg;  
 Xaa<sup>4</sup> is Glu, Gln, Ala, Thr, Ser, and Gly; and,  
 Xaa<sup>5</sup> is Gly-OH or is absent;

into said mammal such that it is immunologically isolated from the mammal's immune system and secretes a protein of SEQ ID NO 1 into said patient.

Preferred proteins of SEQ ID NO 1 are those in which Xaa<sup>1</sup> is Ala, Xaa<sup>2</sup> is Glu, Xaa<sup>3</sup> is Lys. Xaa<sup>4</sup> is Glu, and Xaa<sup>5</sup> is Gly-OH. More preferred are those in which Xaa<sup>1</sup> is Ala, Gly, Val, Thr, and Ile; Xaa<sup>2</sup> is Glu, Gln, Ala, Thr, Ser.

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and Gly; Xaa<sup>3</sup> is Lys, and Arg; Xaa<sup>4</sup> is Glu, Gln, Ala, Thr, Ser, and Gly; and, Xaa<sup>5</sup> is Gly-OH or is absent. Another preferred group are those in which Xaa<sup>1</sup> is Val and Xaa<sup>3</sup> is Lys. A further preferred group of proteins are those wherein 5 Xaa<sup>1</sup> is Ala or Val, Xaa<sup>2</sup> Glu, Xaa<sup>3</sup> is Lys or Arg, Xaa<sup>4</sup> is Glu, and Xaa<sup>5</sup> Gly-OH or is absent. Yet another preferred group are those in which Xaa<sup>1</sup> is Ala, Xaa<sup>2</sup> Glu, Xaa<sup>3</sup> is Lys, Xaa<sup>4</sup> is Glu, and Xaa<sup>5</sup> Gly-OH. Still another preferred group is when Xaa<sup>1</sup> is Val, Xaa<sup>2</sup> Glu, Xaa<sup>3</sup> is Lys, Xaa<sup>4</sup> is Glu, and 10 Xaa<sup>5</sup> Gly-OH.

Nucleotide sequences encoding any one of the polypeptides of SEQ ID NO 1 may be prepared by a variety of means readily apparent to those skilled in the art. Wholly synthetic nucleotide sequences or semi-synthetic sequences 15 derived in part from a natural GLP-1 gene may be used. Owing to the natural degeneracy of the genetic code, the skilled artisan will recognize that a sizable yet definite number of nucleotide sequences may be constructed which encode the proteins of SEQ ID NO 1. A synthetic DNA sequence encoding a 20 GLP-1-based protein of SEQ ID NO 1 may be prepared by techniques well known in the art in substantial accordance with the teachings of Brown, *et al.* (1979) *Methods in Enzymology*, Academic Press, N.Y., Vol. 68, pgs. 109-151. The DNA sequence may be generated using conventional DNA 25 synthesizing apparatus such as an Applied Biosystems Model 380A or 380B DNA synthesizer (commercially available from Applied Biosystems, Foster City, California). Commercial services are also available for the construction of such nucleotide sequences based on the amino acid sequence.

30 In one preferred embodiment of the invention as exemplified herein, the coding sequence for a protein of SEQ ID NO 1 is the following:

5' - CAT GCT GAA GGG ACC TTT ACC AGT GAT GTA AGT TCT TAT TTG  
GAA GGC CAA GCT GCC AAG GAA TTC ATT GCT TGG CTG GTG AAA  
35 GGC CGA GGA - 3' **(SEQ ID NO 2).**

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This sequence encodes the following protein of SEQ ID NO 1:

H<sub>2</sub>N- His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu  
Glu Gly Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys  
Gly Arg Gly -OH **(SEQ ID NO 3).**

5 In another preferred embodiment of the invention as exemplified herein, the coding sequence for a protein of SEQ ID NO 1 is the following:

5' - CAT GTT GAA GGG ACC TTT ACC AGT GAT GTA AGT TCT TAT TTG  
GAA GGC CAA GCT GCC AAG GAA TTC ATT GCT TGG CTG GTG AAA  
10 GGC CGA GGA - 3' **(SEQ ID NO 4).**

This sequence encodes the following protein of SEQ ID NO 1:

H<sub>2</sub>N- His Val Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu  
Glu Gly Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys  
Gly Arg Gly -OH **(SEQ ID NO 5).**

15 Coding regions for SEQ ID NO 1 may be fused to a leader sequence which signals the cell to secrete a precursor peptide of SEQ ID NO 1 that is subsequently processed by the cell during the secretion process to a protein of SEQ ID NO 1. Many such leader sequences are known in the art. One well known and preferred leader sequence is the hybrid tissue plasminogen activator/protein C prepropeptide described in Berg et al. *Biochem. Biophys. Res. Commun.* 179: 1289-1296 (1991). Typically nucleotide sequences encoding precursor peptides of SEQ ID NO 1 are flanked by linker DNA to facilitate enzymatic ligation into expression vectors as is later exemplified herein.

Once a suitable coding sequence of SEQ ID NO 1 is constructed and optionally fused and flanked by an appropriate leader sequence and linker DNA, the construct is 30 ligated into an expression vector which is then introduced into an appropriate cell line. Construction of suitable vectors containing the desired coding and control sequences may be constructed by standard ligation techniques. Isolated

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plasmids or nucleotide fragments are cleaved, tailored, and religated in the manner necessary to achieve the desired plasmids.

To effect the expression of a polypeptides of SEQ ID NO 1, one ligates a nucleotide sequence encoding the polypeptide into an appropriate recombinant nucleotide expression vector through the use of appropriate enzymes. The nucleotide sequence encoding a polypeptide of SEQ ID NO 1 is designed to possess restriction endonuclease cleavage sites at either end of the DNA to facilitate isolation from and integration into these amplification and expression plasmids. The coding sequence may be readily modified by the use of synthetic linkers to facilitate the incorporation of the coding sequence into the desired cloning vectors by techniques well known in the art. The particular endonucleases employed will be dictated by the restriction endonuclease cleavage pattern of the parent expression vector to be employed. The choice of restriction sites are chosen so as to properly orient the coding sequence such that it is properly associated with the promoter and ribosome binding site of the expression vector, both of which are functional in the host cell in which a compound of SEQ ID NO 1 is to be expressed.

In addition to the desired nucleotide sequence which will code for the therapeutic protein, the expression vector may contain several other functional elements. One such element is the promoter and upstream regulatory sequences which control the level of expression of the protein of interest. Some expression vectors contain promoters and regulatory sequences which normally regulate transcription of cellular genes. One such promoter is the mouse metallothionein-I promoter which has been shown to function both *in-vitro* and *in-vivo* (Palmiter et al., *Nature* 300: 611 - 615, 1982). In addition, promoters and regulatory sequences from viruses are frequently used in expression vectors (Dijkema et al., *EMBO J.* 4, 471, 1985; Gorman et al., *Proc. Natl. Acad. Sci.* 79: 6777, 1982; Boshart, et al. *Cell* 41:

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521, 1985). Although, Verma et al. have shown that the retrovirus promoter and enhancer sequences do not function for long periods of time *in-vivo*. In addition, the expression vector also carries an origin of replication as well as marker sequences which are capable of providing phenotypic selection in transformed cells.

10 Because the proteins useful in the present invention do not require post-translational processing mechanisms other than enzymatic removal of the propeptide leader sequence, many stable human cell lines are consistent with the practice of the invention. One preferred cell line is the human embryonal kidney cell line 293, available from the permanent collection of the American Type Culture Collection.

15 A number of well known methods exist for introducing the genetic material into target cells such as chemical (calcium phosphate precipitation), physical (electroporation and microinjection), and viral methods (adenovirus, retrovirus, and adeno-associated virus) (Methods for Gene Transfer, Gene Therapy, Mary Ann Liebert, Inc., 1994). All such methods are consistent with the practice of the present invention. The techniques of transforming mammalian cells with the aforementioned vector types are well known in the art and may be found in such general references as Maniatis, et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York or Current Protocols in Molecular Biology, Vol. 1, (1988), Wiley Interscience, and supplements.

30 Stable transformed cell lines that express proteins of SEQ ID NO 1 must then be implanted into the individual in need of such treatment. Because such transformed cell lines generally will be histologically incompatible with the individuals receiving them, the cells must be protected from the recipient's immune system. One way of protecting the implanted cells is by masking them with F(ab')<sub>2</sub> fragments specific for HLA class I antigens. Immunological masking

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methods are well known in the art. For example, see Faust et al. *Science* 252:1700-1702 (1991). Other means for protecting the implanted cells from the recipient's immune system are consistent with this invention. Such methods include but are 5 not limited to encapsulation in semi-permeable membranes (Lanza et al. *Diabetes* 41: 1503 - 1510) and through the use of immunosuppressants (Rynasiewicz et al., *Diabetes* 31: 92 - 108, 1982).

By way of illustration, the following examples are 10 provided to help describe how to make and practice the various embodiments of the invention. These examples are in no way meant to limit the scope of the invention.

Example 1

Construction of Intermediate

15 Plasmid pLP53-tLB+GLP-1

A. Preparation of BglII-Mung Bean-AvrII Digested pLP53-tLB

The plasmid pLP53-tLB was isolated from *E. coli* K12 AG1 (on deposit under terms of the Budapest Treaty and made part of the permanent stock culture collection of the 20 Northern Regional Research Laboratories (NRRL), agricultural Research Service, U.S. Dept. of Agriculture, Peoria, IL 61604 under accession number NRRL B-18714) using the Plasmid Purification Midi Kit. (Qiagen, Inc., 9600 DeSoto Avenue, Chatsworth, CA 91311).

25 Sixty  $\mu$ l (approximately 20  $\mu$ g) of pLP53-tLB DNA was digested with 2  $\mu$ l (20 units) of BglII in a 70  $\mu$ l reaction volume containing 50mM Tris-HCl (pH 8.0), 10mM MgCl<sub>2</sub>, and 100mM NaCl. The sample was incubated at 37°C for one hour. 30 17.5  $\mu$ l of 5x stop mix (25% glycerol, 2% SDS, 0.05% bromophenol blue, 0.05% xylene cyanol in water) was added and then the reaction was heated at 70°C for 15-20 minutes to inactivate the restriction enzyme. The mixture was spin dialyzed using G-50 Sephadex Quick Spin columns (Boehringer Mannheim Corporation, P.O. Box 50414, 9115 Hague Road,

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Indianapolis, IN 46250-0414) to remove the reaction components.

The 5' protruding ends created by cleavage with BglII were removed using Mung Bean Nuclease. The BglII digested pLP53-tLB DNA was incubated with 0.3  $\mu$ l (approximately 3.3 units) of Mung Bean Nuclease in a 100  $\mu$ l reaction volume containing 10mM Tris-HCl (pH 7.9 at 25°C), 10mM MgCl<sub>2</sub>, 50mM NaCl, 1mM DTT, and 1mM ZnSO<sub>4</sub>. The reaction was allowed to proceed for 30 minutes at 30°C. One  $\mu$ l of 1% SDS was added to inactivate the nuclease. Due to an incomplete BglII digest, the digested and undigested DNA was separated by gel electrophoresis. Ten  $\mu$ l of gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water) was added to the reaction. The reaction was loaded into the preparative well of a 1.5% NuSieve GTG agarose (FMC Bioproducts, 191 Thomaston Street, Rockland, ME 04841)/TAE buffer gel and then electrophoresed. The gel was stained with ethidium bromide and the DNA was visualized by ultraviolet light. The digested DNA band was excised with a scalpel and placed into two micro-tubes. The DNA was purified from the low melting point agarose using the Wizard PCR Preps DNA Purification System (Promega, 2800 Woods Hollow Road, Madison, WI 53711-5399).

One hundred  $\mu$ l of BglII-Mung Bean digested pLP53-tLB DNA was further digested with 4  $\mu$ l (approximately 16 units) of AvrII in a reaction volume of 120  $\mu$ l containing 10mM Tris-HCl (pH 7.9 at 25°C), 10mM MgCl<sub>2</sub>, 50mM NaCl, 1mM DTT. The sample was incubated at 37°C for 30 minutes. To prevent recircularization, the BglII-Mung Bean-AvrII digested pLP53-tLB DNA was dephosphorylated (removal of 5' phosphate groups) by the addition of 2  $\mu$ l (2 units) of calf intestinal alkaline phosphatase to the reaction. The sample was incubated at 37°C for an additional 30 minutes. Twenty-four  $\mu$ l of 5x stop mix was added. The sample was heated at 70°C for 15-20 minutes to inactivate the enzymes and then spin dialyzed using G-50 Sephadex Quick Spin columns (Boehringer Mannheim Corporation, P.O. Box 50414, 9115 Hague Road,

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Indianapolis, IN 46250-0414) in order to remove the AvrII produced small DNA fragments. The DNA was precipitated by addition of 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol. This mixture was mixed  
5 thoroughly and then chilled to -20°C. The precipitate was collected by centrifugation for 30 minutes. The supernatant was discarded and the pellet was washed with 700 µl of cold 70% ethanol. The sample was centrifuged for 15 minutes. The supernatant was discarded, the DNA pellet was dried and then  
10 resuspended in 25 µl of water.

B. Preparation of GLP-1 Linker

The following single stranded DNA segments were conventionally synthesized by methods well known in the art  
15 on an automated DNA synthesizer (model 394 Applied Biosystems 850 Lincoln Center Drive, Foster City, CA 94404-1128) using β-cyanoethyl phosphoramidite chemistry.

GLP-1.1

20 5' - GACATGCTGA AGGGACCTTT ACCAGTGATG TAAGTTCTTA TTTGGAAGGC  
CAAGCTGCCA AGGAATTCA TGCTTGGCTG GTGAAAGGCC GAGGATAGGG  
ATCCC - 3'  
**(SEQ ID NO 6)**

GLP-1.2

25 5' - CTAGGGGATC CCTATCCTCG GCCTTCACC AGCCAAGCAA TGAATTCCCTT  
GGCAGCTTGG CCTTCCAAAT AAGAACTTAC ATCACTGGTA AAGGTCCCTT  
CAGCATGTC - 3'  
**(SEQ ID NO 7)**

30 GLP-1.1 and GLP-1.2 are complementary DNA molecules. The synthetic DNA molecules were dissolved in water and stored at less than 0°C.

To anneal the DNA strands, approximately 92.7 pmoles each of GLP-1.1 and GLP-1.2 were mixed in 50mM Tris-HCl (pH 7.4) and 10mM MgCl<sub>2</sub> in a total volume of 80 µl and  
35 boiled for 5 minutes. The mixture was slowly brought to room temperature and then transferred to 4°C overnight. This process allowed the two complementary strands to anneal and

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form the double stranded DNA linker known as GLP-1. The linker was stored at -20°C. In order to be able to ligate into the dephosphorylated BglII-Mung Bean-AvrII digested pLP53-tLB DNA segment, the GLP-1 linker must have 5' phosphate groups. The phosphate groups were added by the use of the enzyme T4 polynucleotide kinase. The kinase reaction contained 80 µl of the GLP-1 linker, 0.33µM ATP, 70mM Tris-HCl (pH 7.6), 10mM MgCl<sub>2</sub>, 100mM KCl, 1mM β-mercaptoethanol and 37.2 µl (372 units) of T4 polynucleotide kinase. The reaction was incubated at 37°C for 30 minutes. Sixteen µl of 500mM EDTA was added to stop the reaction. The reaction was extracted once with a mixture of phenol:chloroform:isoamyl alcohol (25:24:1) followed by an extraction with chloroform:isoamyl alcohol (24:1). One hundred µl of the aqueous layer was spin dialyzed using G-50 Sephadex Quick Spin columns (Boehringer Mannheim) in order to remove the reaction components. The DNA was precipitated by addition of 0.1 volume of 3M sodium acetate (pH 5.2), 0.1 volume of 100mM MgCl<sub>2</sub> and 2.5 volumes of absolute ethanol. This mixture was mixed thoroughly and then chilled at -20°C. The precipitate was collected by centrifugation for 30 minutes. The supernatant was discarded and the pellet was washed with 700 µl of cold 70% ethanol. The sample was centrifuged for 15 minutes. The supernatant was discarded, the DNA pellet was dried and then resuspended in 10 µl of water.

#### C. Final Construction of pLP53-tLB+GLP-1

The DNA prepared in Example 1A was ligated with linker GLP-1. Two µl of DNA prepared in Example 1A and 4 µl of GLP-1 linker were ligated in a reaction that contained 2 µl (2 units) of T4 DNA ligase, 50mM Tris-HCl (pH 7.6), 10mM MgCl<sub>2</sub>, 1mM ATP, 1mM DTT, and 50% (w/v) polyethylene glycol-8000 in a total volume of 10 µl. The mixture was incubated at 16°C for 16 hours. The ligation was used to transform *E. coli* K12 INVαF' cells as generally described below.

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D. Transformation Procedure

Frozen competent *E. coli* K12 INVαF' cells were obtained from Invitrogen (3985 B Sorrento Valley Boulevard, San Diego, CA 92121). Two  $\mu$ l of 0.5M  $\beta$ -mercaptoethanol were 5 added to 50  $\mu$ l of thawed competent cells. About 1-2  $\mu$ l of the ligation reaction was mixed with the cells. The cell-DNA mixture was incubated on ice for 30 minutes, heat-shocked at 42°C for exactly 30 seconds and then chilled on ice for 2 minutes. The cell-DNA mixture was diluted into 450 $\mu$ l of SOC 10 media (2% tryptone, 0.05% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl<sub>2</sub>, 10mM MgSO<sub>4</sub>, 20mM glucose in distilled water) and incubated at 37°C for one hour in a rotary shaker set at about 225 rpm. Aliquots of up to 200  $\mu$ l were plated on TY- 15 agar plates (1% tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% agar, pH 7.4) containing 100 $\mu$ g/ml ampicillin and then incubated at 37°C until colonies appear.

E. DNA Isolation

Following transformation, ampicillin resistant 20 cells were picked and inoculated into 3 ml of TY broth (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) containing 100 $\mu$ g/ml ampicillin. These cultures were grown for about 16 hours at 37°C with aeration. Plasmid DNA was isolated from 25 cultures using Wizard Minipreps obtained from Promega (2800 Woods Hollow Road, Madison, WI 53711-5399). Recombinant plasmids were identified by digestion with restriction endonucleases followed by gel electrophoresis analysis.

To obtain larger amounts of pLP53-tLB+GLP-1 plasmid DNA, large scale isolation was performed using the Plasmid 30 Purification Midi Kit (Qiagen, Inc.).

Example 2

Construction of pGT-h+tLB+GLP-1

A. Preparation of BclI Digested pGT-h

The plasmid pGT-h was isolated from *E.coli* K12 GM48 35 (on deposit under terms of the Budapest Treaty and made part

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of the permanent stock culture collection of the NRRL under accession number B-18592 using the Plasmid Purification Midi Kit (Qiagen, Inc.).

Ten  $\mu$ g (37.5  $\mu$ l) of pGT-h DNA was digested to completion with 2  $\mu$ l (20 units) of BclI in a 45  $\mu$ l reaction volume containing 50mM Tris-HCl (pH 8.0), 10mM MgCl<sub>2</sub>, 50mM NaCl. The sample was incubated at 50°C for 1 hour. Eleven  $\mu$ l of 5x stop mix was added to the reaction mixture. The mixture was heated at 70°C for 15-20 minutes to inactivate the restriction enzyme and then spin dialyzed using G-50 Sephadex Quick Spin columns (Boehringer Mannheim) in order to remove the reaction components. The DNA was precipitated by adding 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol. This mixture was mixed thoroughly and then chilled at -20°C. The precipitate was collected by centrifugation for 30 minutes. The supernatant was discarded and the pellet was washed with 700  $\mu$ l of cold 70% ethanol. The sample was centrifuged for 15 minutes. The supernatant was discarded, the DNA pellet was dried and then resuspended in 20  $\mu$ l of water.

Calf intestinal alkaline phosphatase was used to remove the 5' phosphate groups from the DNA segment in order to prevent recircularization of the pGT-h. Ten  $\mu$ l of the BclI digested pGT-h DNA was treated with 1  $\mu$ l (1 unit) of calf intestinal alkaline phosphatase in a 15  $\mu$ l reaction containing 50mM Tris-HCl (pH 8.5 at 20°C) and 0.1mM EDTA. The reaction was allowed to proceed for 45 minutes at 37°C. The phosphatase was inactivated by the addition of 1 $\mu$ l of 500mM EDTA and then heating at 65°C for 10 minutes. The reaction volume was increased to 100 $\mu$ l with water and then extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) followed by extraction with chloroform: isoamyl alcohol (24:1). The DNA was recovered from the aqueous layer by the addition of 0.1 volumes of 3M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol. The mixture was mixed thoroughly and then chilled at -20°C. The precipitate was collected by centrifugation for 30 minutes. The supernatant

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was discarded and the pellet was washed with 700 µl of cold 70% ethanol. The sample was centrifuged for 15 minutes. The supernatant was discarded, the DNA pellet was dried and then resuspended in 25 µl of water.

5

B. Preparation of pLP53-tLB+GLP-1 BamHI Fragment

Thirty-five µl (10.6 µg) of pLP53-tLB+GLP-1 DNA, prepared in Example 1, was digested with 0.5 µl (25 units) of BamHI in a 40 µl reaction volume containing 50mM Tris-HCl (pH 8.0), 10mM MgCl<sub>2</sub>, and 100mM NaCl. The reaction was allowed to proceed at 37°C for one hour. Five µl of gel loading dye was added to the reaction. The reaction was loaded into the preparative well of a 4% NuSieve GTG agarose/TAE buffer gel. The DNA was electrophoresed for about one hour at 70 constant volts. The gel was stained with ethidium bromide and then the DNA was visualized by ultraviolet light. The desired 213 base pair DNA band was excised using a scalpel. The DNA was purified from the low melting point agarose using Wizard PCR preps (Promega).

20

C. Final Construction of pGT-h+tLB+GLP-1

The DNA prepared in Example 2A was ligated with DNA prepared in Example 2B. One µl of DNA from Example 2A and 10.5 µl of DNA from Example 2B were ligated in a reaction that contained 2 µl (2 units) of T4 DNA ligase, 50mM Tris-HCl (pH 7.6), 10mM MgCl<sub>2</sub>, 1mM ATP, 1mM DTT, and 50% (w/v) polyethylene glycol-8000 in a total volume of 20 µl. The mixture was incubated at 16°C for 16 hours. The ligation reaction was used to transform *E. coli* K12 INVαF' as described in Example 1D. Plasmid DNA was isolated from ampicillin resistant cultures as described in Example 1E.

To obtain larger amounts of pGT-h+tLB+GLP-1 plasmid DNA for the purpose of transfection of mammalian cells, large scale isolation was performed using the alkaline lysis method.

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Example 3

Construction of pGT-h+tLB+Val18GLP-1

The plasmid pGT-h+tLB+Val18GLP-1 was constructed substantially in accordance with Examples 1 and 2. To 5 accommodate the change in the amino acid sequence (Ala to Val at Xaa<sup>1</sup> in SEQ ID NO 1) the following coding sequences were substituted for those described in Example 1B:

Val18GLP-1.1

10 5' - GACATGTTGA AGGGACCTTT ACCAGTGATG TAAGTTCTTA TTTGGAAGGC  
CAAGCTGCCA AGGAATTCCAT TGCTTGGCTG GTGAAAGGCC GAGGATAGGG  
ATCCC - 3' (SEQ ID NO 8)

Val18GLP-1.2

15 5' - CTAGGGGATC CCTATCCTCG GCCTTCACC AGCCAAGCAA TGAATTCCCTT  
GGCAGCTTGG CCTTCCAAAT AAGAACTTAC ATCACTGGTA AAGGTCCCTT  
CAACATGTC - 3' (SEQ ID NO 9)

Example 4

Construction of Intermediate

20 Plasmid pM100-neo

A. Preparation of EcoRI Digested pM100

The plasmid pM100 (pOK12) was isolated from *E. coli* K12 RR1ΔM15 using Magic Minipreps (Promega). See Vieira and Messing, Gene 100:189-94, 1991). Forty μl of pM100 DNA was 25 digested to completion with 3 μl of EcoRI in a reaction volume of 50 μl containing 50mM Tris-HCl (pH 8.0), 10mM MgCl<sub>2</sub>, and 100mM NaCl. The sample was incubated at 37°C for 2 hours. The digested DNA was precipitated with ethanol. The final DNA pellet was resuspended in 30 μl of water.

30

B. Preparation of pBK-neo EcoRI Fragment

The plasmid pBK-neo 1 (described in U S Patent No: 5,550,036, herein incorporated by reference, and available from the American Type Culture Collection under terms of the

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Budapest Treaty via Accession No. Atcc 37224) was isolated from *E.coli* K12 HB-1 using the large scale alkaline lysis method. Three  $\mu$ l (26.3  $\mu$ g) of pBK-neo DNA was digested to completion with 7  $\mu$ l (15 units) of EcoRI in a reaction volume of 200  $\mu$ l containing 50mM Tris-HCl (pH 8.0), 10mM MgCl<sub>2</sub>, and 100mM NaCl. The sample was incubated at 37°C. Fifty  $\mu$ l of the reaction was loaded into the preparative well of an agarose/TAE buffer gel and then electrophoresed. The desired 4.2 Kb DNA band was isolated from the agarose by electrophoresing onto DEAE-cellulose membrane. Following elution from the DEAE-cellulose membrane, the DNA was precipitated with ethanol. The final DNA pellet was resuspended in 30  $\mu$ l of water.

15 C. Final Construction of pM100-neo

The DNA prepared in Example 4A was ligated with DNA prepared in Example 4B. Two  $\mu$ l of DNA from Example 4A and 2  $\mu$ l of DNA from Example 4B were ligated in a reaction that contained 1  $\mu$ l (1 unit) of T4 DNA ligase, 50mM Tris-HCl (pH 7.6), 10mM MgCl<sub>2</sub>, 1mM ATP, 1mM DTT, and 50% (w/v) polyethylene glycol-8000 in a total volume of 16  $\mu$ l. The mixture was incubated at 16°C for 16 hours. Ten  $\mu$ l of the ligation reaction was used to transform *E. coli* K12 RR1ΔM15 as described in Example 5D. Aliquots of up to 200  $\mu$ l were plated on TY-agar plates (1% tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% agar, pH 7.4) containing kanamycin and then incubated at 37°C until colonies appear.

D. DNA Isolation

Following transformation, kanamycin resistant cells were picked and inoculated into 5 ml of TY broth (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) containing kanamycin. These cultures were grown for about 8 hours at 37°C with aeration. Plasmid DNA was isolated from cultures using Magic Minipreps obtained from (Promega). Recombinant plasmids were identified by digestion with restriction endonucleases followed by gel electrophoresis analysis.

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To obtain larger amounts of pM100-neo plasmid DNA, large scale isolation was performed using the Plasmid Purification Kit (Qiagen, Inc.).

Example 5

5

Construction of pMT-h+tLB+Val8GLP-1

A. Preparation of Bst1107I-BclI Digested pGT-h

The plasmid pGT-h was isolated as described in substantial accordance with Example 2A. Three µg (4.6 µl) of pGT-h DNA was digested to completion with 1 µl (8 units) of Bst1107I in a 10 µl reaction volume containing 10mM Tris-HCl, 10mM MgCl<sub>2</sub>, 100mM KCl (pH 8.5 at 37°C). The sample was incubated at 37°C for 1 hour. The Bst1107I digested pGT-h DNA was purified from the reaction components using the Wizard DNA Clean-Up System (Promega). The Bst1107I digested pGT-h DNA was concentrated in a 6.7 µl volume using a microcon 50 (Amicon, Inc. 72 Cherry Hill Drive, Beverly, MA 01915).

The 6.7 µl of Bst1107I digested pGT-h DNA was further digested with 1 µl (10 units) of BclI in a reaction volume of 10 µl containing 50mM Tris-HCl (pH 8.0), 10mM MgCl<sub>2</sub>, 50mM NaCl. The sample was incubated at 50°C for 1 hour. To prevent any possible recircularization, the Bst1107I-BclI digested pGT-h was dephosphorylated (removal of 5' phosphate groups) by the addition of 1 µl (1 unit) of calf intestinal phosphatase to the sample. The sample was incubated at 37°C for 30 minutes. Gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water) was added to the reaction. The reaction was loaded into the preparative well of a 1% SeaPlaque GTG agarose/TAE buffer gel and then electrophoresed. The gel was stained with ethidium bromide and then the DNA was visualized by ultraviolet light. The desired 6.2 Kb DNA band was excised with a scalpel and placed into a micro-tube. The DNA was purified from the low melting point agarose using the Wizard PCR Preps DNA Purification System (Promega).

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B. Preparation of pM100-neo EcoRV-BglII Fragment

Five µg (3.8 µl) of pM100-neo DNA constructed in Example 4 was digested to completion with 1 µl (10 units) of EcoRV in a 10 µl reaction volume containing 50mM Tris-HCl (pH 8.0), 10mM MgCl<sub>2</sub>, 50mM NaCl. The reaction was incubated at 37°C for 1 hour. The EcoRV digested pM100-neo was further digested with 1 µl (10units) of BglII in a 20 µl reaction volume containing 50mM Tris-HCl (pH 8.0), 10mM MgCl<sub>2</sub>, 50mM NaCl. The sample was incubated at 37°C for 1 hour. Gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water) was added to the reaction. The reaction was loaded into the preparative well of a 1% SeaPlaque GTG agarose/TAE buffer gel and then electrophoresed. The gel was stained with ethidium bromide and then the DNA was visualized by ultraviolet light. The desired 1.8 Kb DNA band was excised with a scalpel and placed into a micro-tube. The DNA was purified from the low melting point agarose using the Wizard PCR Preps DNA Purification System (Promega).

C. Final Construction of pMT-h+tLB+Val8GLP-1

The DNA prepared in Example 5A was ligated with the DNA prepared in Example 5B and the DNA prepared in Example 3 (pLP53-tLB+Val8GLP-1 BamHI fragment). Four µl of DNA from Example 5A, 3 µl of DNA from Example 5B and 12 µl of DNA prepared in Example 3 (pLP53-tLB+Val8GLP-1 BamHI fragment) were ligated in a reaction that contained 1 µl (1 unit) of T4 DNA ligase, 50mM Tris-HCl (pH 7.6), 10mM MgCl<sub>2</sub>, 1mM ATP, 1mM DTT, and 50% (w/v) polyethylene glycol-8000 in a total volume of 25 µl. The mixture was incubated at 16°C for 16 hours.

Frozen competent E.coli K12 DH5α cells were transformed using about 3-4 µl of the ligation reaction in substantial accordance with Example 1D, and the plasmid DNA was isolated in substantial accordance with Example 1E.

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Example 6

Construction of Stable Cell lines

Plasmid DNA, either pGT-h+tLB+GLP-1, pGT-h+tLB+val8GLP-1, or pMT-h+tLB+val8GLP-1 was transfected into 5 human embryonic kidney 293 cells using the stable CaPO<sub>4</sub> method contained in the Mammalian Transfection Kit available from Stratagene (11011 North Torrey Pines Road, LaJolla, CA 92037). Selection was achieved by the addition of 300µg/ml of hygromycin B (Eli Lilly and Company Indianapolis, IN 10 46285) to the culture medium. Monoclonal cell lines were expanded and screened for the ability to secrete the corresponding protein of SEQ ID NO 1 into the culture medium. The presence of biologically active GLP-1(7-37)-based protein in the culture medium was determined by measuring the amount 15 of luciferase enzyme present in a biological system that expressed luciferase enzyme following stimulation with GLP-1.

Example 7

Implantation

The transformed 293 cell were cultured then 20 surgically transplanted under the kidney capsule of 8 week old Zucker Diabetic Fatty (ZDF/Gmi<sup>TM</sup>-fa/fa) male rats. Under isofurane anesthesia, a dorsal incision was made just posterior to the diaphragm, and using a rib spreader, the kidney was exposed. Approximately 20 million transformed 293 25 cells, in 200 µl of Hank's buffer, were injected just under the kidney capsule using a 23 gauge blunt needle. The incision was sutured and protected from chewing with wound clips.

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We Claim:

1. A method of treating Type I or Type II diabetes in a mammal in need thereof comprising implanting a cell line transformed with a vector comprising a promotor driving expression of a DNA sequence encoding a protein of the formula:

His-Xaa<sup>1</sup>-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Xaa<sup>2</sup>-Gly-Gln-Ala-Ala-Xaa<sup>3</sup>-Xaa<sup>4</sup>-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Xaa<sup>5</sup> (SEQ ID NO 1)

10 wherein

Xaa<sup>1</sup> is Ala, Gly, Val, Thr, and Ile;

Xaa<sup>2</sup> is Glu, Gln, Ala, Thr, Ser, and Gly;

Xaa<sup>3</sup> is Lys, and Arg;

Xaa<sup>4</sup> is Glu, Gln, Ala, Thr, Ser, and Gly; and,

Xaa<sup>5</sup> is Gly-OH or is absent;

15 into said mammal such that it is immunologically isolated from the mammal's immune system and secretes a protein of SEQ ID NO. 1 into said patient.

20 2. A method of treating Type I or Type II diabetes in a mammal in need thereof comprising implanting a cell line transformed with a vector comprising a promotor driving expression of a DNA sequence encoding a protein of SEQ ID NO 1 into said mammal wherein said mammal is under 25 immunosuppression therapy.

3. The method of Claims 1 or 2 wherein Xaa<sup>1</sup> is Ala or Val, Xaa<sup>2</sup> Glu, Xaa<sup>3</sup> is Lys or Arg, Xaa<sup>4</sup> is Glu, and Xaa<sup>5</sup> Gly-OH or is absent.

30

4. The method of Claims 1 or 2 wherein Xaa<sup>1</sup> is Ala, Xaa<sup>2</sup> Glu, Xaa<sup>3</sup> is Lys, Xaa<sup>4</sup> is Glu, and Xaa<sup>5</sup> Gly-OH.

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5. The method of Claims 1 or 2 wherein Xaa<sup>1</sup> is Val, Xaa<sup>2</sup> Glu, Xaa<sup>3</sup> is Lys, Xaa<sup>4</sup> is Glu, and Xaa<sup>5</sup> Gly-OH.

5 6. The method of Claims 1 or 2 wherein the promotor is a viral promotor.

10 7. The method of Claims 1 or 2 wherein the promotor is a metallothionein promotor.

15 8. The method of Claims 1 or 2 wherein the DNA coding sequence is

5' - CAT GCT GAA GGG ACC TTT ACC AGT GAT GTA AGT TCT TAT TTG  
GAA GGC CAA GCT GCC AAG GAA TTC ATT GCT TGG CTG GTG AAA  
GGC CGA GGA - 3'. **(SEQ ID NO 2)**

9. The method of Claims 1 or 2 wherein the DNA coding sequence is

5' - CAT GTT GAA GGG ACC TTT ACC AGT GAT GTA AGT TCT TAT TTG  
GAA GGC CAA GCT GCC AAG GAA TTC ATT GCT TGG CTG GTG AAA  
GGC CGA GGA - 3'. **(SEQ ID NO 4)**

20 10. The method of Claims 1 or 2 wherein the cell line is the human embryonal kidney cell line 293 transformed with a vector selected from the group consisting of pGT-h+tLB+GLP-1, pGT-h+tLB+Val8GLP-1, or pMT-h+tLB+Val8GLP-1.

30 11. The method of Claims 1 or 2 wherein the cell line is the human embryonal kidney cell line 293 transformed with the vector pGT-h+tLB+GLP-1.

12. The method of Claims 1 or 2 wherein the cell line is the human embryonal kidney cell line 293 transformed with the vector pGT-h+tLB+Val8GLP-1.

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13. A method of treating Type I or Type II diabetes in a mammal in need thereof comprising injecting an expression vector of any one of Claims 1 to 12 directly into 5 the mammal such that the expression vector is incorporated into a cell of the mammal and secretes a protein of SEQ ID NO. 1.

14. A stable transformed cell line of any one of 10 Claims 1 to 12.

15. A vector of any one of Claims 1 to 12.

16. A method of treating Type I or Type II 15 diabetes in a mammal in need thereof comprising implanting a cell line transformed with a vector comprising a promotor driving expression of a DNA sequence encoding a protein of the formula:

20 His-Xaa<sup>1</sup>-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-  
Leu-Xaa<sup>2</sup>-Gly-Gln-Ala-Ala-Xaa<sup>3</sup>-Xaa<sup>4</sup>-Phe-Ile-Ala-Trp-Leu-  
Val-Lys-Gly-Arg-Xaa<sup>5</sup> (SEQ ID NO 1)

wherein

Xaa<sup>1</sup> is Ala, Gly, Val, Thr, and Ile;

Xaa<sup>2</sup> is Glu, Gln, Ala, Thr, Ser, and Gly;

25 Xaa<sup>3</sup> is Lys, and Arg;

Xaa<sup>4</sup> is Glu, Gln, Ala, Thr, Ser, and Gly; and,

Xaa<sup>5</sup> is Gly-OH or is absent;

into said mammal such that it is immunologically isolated from the mammal's immune system substantially as hereinbefore 30 described with reference to any one of the Examples.

17. A vector encoding a protein of SEQ ID NO 1 substantially as hereinbefore described with reference to any one of the Examples.

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18. A stable mammalian cell line transformed with  
a vector capable of secreting a protein of SEQ ID NO 1  
substantially as hereinbefore described with reference to any  
one of the Examples.

5



# DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

- Declaration Submitted with Initial Filing  
 Declaration Submitted after Initial Filing

Attorney Docket Number	X-9872
First Named Inventor	Tracy Leitner Borts
<b>COMPLETE IF KNOWN</b>	
Application Number	
Filing Date	
Group Art Unit	
Examiner Name	KAREN M. HAUDA

As a below named Inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole Inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the Invention entitled:

**DIABETES THERAPY**

*(Title of the Invention)*

the specification of which  
 is attached hereto

OR

was filed on  
(MM/DD/YYYY)  as United States Application Number or PCT International

Application Number  and was amended on  
(MM/DD/YYYY)  (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or Inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached YES	Certified Copy Attached NO
9603847.6	GB	02/23/1996	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>

Additional foreign application numbers are listed on a supplemental priority sheet attached hereto:

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional applications(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)	<input type="checkbox"/> Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.
60/012,111	02/06/1996	<input type="checkbox"/>

Please type a plus sign (+) inside this box

2

PTO/SB/01 (8-96) (MODIFIED)

Approved for use through 9/30/98. OMB 0651-0032  
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

## **DECLARATION**

**PRIORITY DATA**  
**(Supplemental Sheet)**

**Additional foreign applications:**

**Additional provisional applications:**

Application Number	Filing Date (MM/DD/YYYY)

#### **Additional U.S. applications:**

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YY)	Parent Patent Number <i>(if applicable)</i>

Please type a plus sign (+) inside this box

PTO/SB/01 (8-96) (MODIFIED)

Approved for use through 9/30/98. OMB 0651-0032  
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

DECLARATION		REGISTERED PRACTITIONER INFORMATION (Supplemental Sheet)	
Name	Registration Number	Name	Registration Number

+
**DECLARATION**

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

<b>U.S. Parent Application Number</b>	<b>PCT Parent Number</b>	<b>Parent Filing Date (MM/DD/YYYY)</b>	<b>Parent Patent Number (if applicable)</b>

Additional U.S. or PCT international application numbers are listed on a supplemental priority sheet attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Name	Reg. #	Name	Reg. #
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Michael T. Bates	34,121	James P. Leeds	35,241
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Additional registered practitioner(s) named on a supplemental sheet attached hereto.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor:  A Petition has been filed for this unsigned inventor

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Additional Inventors are being named on supplement sheet(s) attached hereto.



## DECLARATION

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